

PRELIMINARY AMENDMENT

Title: Narcistatin Prodrugs

United States National Stage of PCT/US03/39067

Amendments to the Specification:

Please amend the paragraph, which appears at page 1, immediately after the heading "RELATED APPLICATION DATA" as follows:

This application is the U.S. national stage of PCT/US03/39067 filed on December 9, 2003, which is based on and claims the benefit of U.S. Provisional Patent Application No. 60/432,219 filed on December 9, 2002-2002, which is incorporated herein in its entirety by this reference.

Please replace the paragraph beginning on page 4, immediately after the heading "DETAILED DESCRIPTION OF THE INVENTION" with the following amended paragraph:

Early experience by one of the inventors in nucleotide chemistry involving phosphate esters and cellular phosphatases combined with recent successes in synthesis of phosphate prodrugs made such an approach most attractive for obtaining a water soluble narciclasine prodrug. (Pettit, G. R. Synthetic Nucleotides, Van Nostrand Reinhold Co: New York, 1972; Pettit, G. R., *et al.*, Anti-Cancer Drug Design 2000, 15, 389-395; Pettit, G. R., *et al.*, Anti-Cancer Drug Design 1995, 10, 243-250; Pettit, G. R., *et al.*, Anti-Cancer Drug Design 2000, 15, 397-403; Saulnier, M. G., *et al.*, Med. Chem. Lett. 1994, 4, 2567-2572; Ueda, Y., *et al.*, Med. Chem. Lett. 1995, 5, 247-252.) However, a selection of the more obvious methods such as POCl₃, or 2-cyanoethylphosphate with dicyclohexylcarbodiimide (DCCI), and various unprotected or ~~protection~~protected (e.g. narciclasine 3,4-acetonide) strategies involving narciclasine (1) led to unpromising mixtures. (Pettit, G. R., *et al.*, Anti-Cancer Drug Design 2000, 15, 389-395; Pettit, G. R., *et al.*, Anti-Cancer Drug Design 1995, 10, 243-250; Taktakishvili, M., *et al.*, Tetrahedron Lett. 2000, 41, 7173-7176; Tener, G. M., J. Amer. Chem. Soc. 1961, 83, 159-168; Scheit, K. H., Nucleotide Analogs, Synthesis and Biological Function; Wiley-Interscience: New York, 1972;

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Khorana, H. G., *et al.*, J. Chem. Soc. 1953, 2257-2260; Khorana, H. G. J. Amer. Chem. Soc. 1954, 76, 3517-3527; Dekker, C. A., *et al.*, J. Amer. Chem. Soc. 1954, 76, 3522-3527; Tener, G. M.; Khorana, H. G., J. Amer. Chem. Soc. 1955, 77, 5348.) Eventually, the inventors examined use of the readily soluble tetrabutylammonium dihydrogen phosphate in pyridine as the phosphate source. Initially, the phosphate failed to couple with narciclasine in the presence of DCCI until three equivalents of *p*-toluenesulfonic acid was employed to promote condensation, at which point precipitation of dicyclohexylurea (DCU) began. When the reaction mixture was heated to 80°C, the pyridinium salt of narciclasine-3,4-cyclic phosphate **3a** (herein designated pyridinium narcistatin), precipitated. Following collection of precipitated DCU and the narcistatin pyridinium salt, the solids were titrated with water to dissolve the cyclic phosphate (**3a**). Concentration of the water fraction afforded the pyridinium salt in 40% yield. The mother liquor was concentrated to a brown oil and added to a large volume of water; an immediate precipitate was observed. The solution was filtered and the filtrate was found to be primarily unreacted narciclasine with some DCU as impurity. The reaction did not go to completion even after prolonged stirring and addition of more reagents.

Please replace the paragraph beginning on page 8, immediately after the subtitle “Pyridinium Narcistatin (3a)” with the following amended paragraph:

Narciclasine **1** (1.0 g, 3.4 mmol) was added to pyridine (50 ml) and the solution was heated to 80°C. Next, tetrabutylammonium-dihydrogen phosphate (5.13 g, 15.11 mmol, 4.4 equiv), dicyclohexylcarbodiimide (5.0, 24.5 mmol, 7.0 equiv) and *p*-toluenesulfonic acid (3.0 g, 15.8 mmol, 4.63 equiv, added slowly) were added. After 2g of the sulfonic acid was added, a precipitate began to separate. The reaction mixture was stirred under argon at 80°C for 2.5 hours. The precipitate was collected and washed with methanol to remove pyridine. The precipitated cyclic phosphate (**3a**) was separated from the DCU by washing with water (200 ml). The aqueous filtrate was concentrated to an off-white solid and dried (vacuum) overnight to yield 0.59 g, 40.4%. The mother liquor was concentrated to a brown oil and water (750 ml)

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added. An immediate precipitate was observed, which was collected and dried to 0.75 g of white solid. The ^1H NMR (DMSO- d_6) showed this material to be recovered starting material with a small amount of DCU impurity. Recrystallization of phosphate **3a** from pyridine-water gave crystals that were used for X-ray crystallography. $[\alpha]^{26}_D = -6.4^\circ$ (c 0.44, DMSO); m.p. 275°C ; ^1H NMR (DMSO- d_6 , 500 MHz) δ 13.66 (s, 1H), 9.00 (s, 1H), 8.60 ppm (m, 3H), 7.9 (t, $J = 7.5$ Hz, 1H), 7.5 (m, 2H), 7.04 (s, 1H), 6.5 (s, 1H), 6.06 (d, $J = 3$ Hz, 2H), 4.42-4.31 (m, 4H), 4.15 (dd, $J_{4,5} = 6.5$ Hz, 1H); ^{13}C NMR (DMSO, 500 MHz) δ 167.7, 152.6, 148.6(2), 145.2, 137.4(2), 133.5, 128.5, 126.9, 125.3, 124.4, 104.3, 102.1, 94.3, 76.9, 76.7, 70.4, 53.9; ^{31}P (DMSO- d_6 , 200 MHz) 20.3 (s, 1P); found by HRAPCI (negative ions) mass spec. 368.0179, calc. for $\text{C}_{14}\text{H}_{11}\text{O}_9\text{NP}$ 368.2164.

Please replace the paragraph beginning on page 11, immediately after the subtitle “Narcistatin (3b)” with the following amended paragraph:

A solution of pyridinium narcistatin (**3a**, 0.05 g) in water (2 ml) was obtained by heating (water bath) at 60°C ~~and allowing the solution.~~ The solution was allowed to cool prior to passing through a column prepared from Dowex 50X8-200 cation exchange resin (hydrogen form). A suspension began to form in the column as the phosphoric acid (**3b**) formed. The column was eluted with water and phosphoric acid **3b** eluted as a milky white suspension. The combined fractions containing phosphoric acid **3b** were freeze dried to afford the product as a colorless solid, (36 mg, 86%); m.p. 175°C (dec.); ^1H NMR (DMSO- d_6 , 300 MHz), δ 13.65 (s, 1H), 9.02 (s, 1H), 7.06 (s, 1H), 6.48 (s, 1H), 6.17 (d, $J_{ab} = 10.2$ Hz, 1H), 6.06 (m, 2H), 4.46-4.30 (m, 3H), 4.18 (m, 1H); calc for $\text{C}_{14}\text{H}_{13}\text{NO}_9\text{P}$ 370.0328; found by HR (APCI) $[\text{M}+\text{H}]^+$ 370.0361.

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Please replace the paragraph beginning on page 12, immediately after the subtitle “Sodium Narcistatin (3d). Procedure B.” with the following amended paragraph:

Narciclasine [1]1 (0.113 g, 0.368 mmol) was added to pyridine (4ml) and the solution heated to 80°C. Next, tetrabutylammonium dihydrogen phosphate (0.075 g, 0.22 mmol, 0.6 equiv.) and dicyclohexylcarbodiimide (0.4 g, 1.93 mmol, 5 equiv.) were added. The reaction mixture was stirred under argon at 80°C for 24 hours. Tetrabutylammonium dihydrogen phosphate (0.185 g) was added followed by DCCI (0.4 g) and the reaction stirred for a further 72 hours. ¹HNMR (DMSO-d₆) of the crude reaction mixture showed complete conversion to product. The reaction was cooled and filtered. Water (100 ml) was added to the mother liquor, which was then filtered to remove any precipitated DCU. The aqueous solution was then concentrated to minimum volume. The solution was then eluted on an ion exchange column of Dowex 50WX8-200 (sodium form) and the UV active fractions were combined and freeze ~~dried~~dried to afford the product as a white solid (0.113 mg, 88%). Comparison of the ¹HNMR of this product in DMSO-d₆ with the narcistatin sodium salt **3d** prepared from the pyridinium narcistatin **3a** by the method outlined above showed them to be identical. This method is more practical and dramatically improves the yield of narcistatin from narciclasine.